

The interplay between human cytomegalovirus and endothelial cells

Citation for published version (APA):

Slobbe-van Drunen, M. E. P. (1998). *The interplay between human cytomegalovirus and endothelial cells*. [Doctoral Thesis, Maastricht University]. Universiteit Maastricht. <https://doi.org/10.26481/dis.19981127ms>

Document status and date:

Published: 01/01/1998

DOI:

[10.26481/dis.19981127ms](https://doi.org/10.26481/dis.19981127ms)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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- The final published version features the final layout of the paper including the volume, issue and page numbers.

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SUMMARY AND GENERAL DISCUSSION

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Human cytomegalovirus (HCMV) infection appears to be involved in several vascular diseases, such as transplantation associated arteriosclerosis, restenosis following coronary angioplasty and atherosclerosis. In this thesis, some aspects of the mechanisms by which this may occur have been studied in vitro using endothelial cells and fibroblasts as a model system. Endothelial cells were chosen because of their strategic location between the blood circulation and the surrounding tissue. The endothelial cell responses were compared with the responses of fibroblasts to HCMV infection

Since diseases in which CMV possibly is involved are often associated with a (pro-) thrombotic state of the vascular wall we first studied the influence of two HCMV strains, AD169 (propagated on fibroblasts) and VHL-E (endothelial cell adapted virus), on membrane perturbation and procoagulant activity on human umbilical vein endothelial cells (HUVEC), microvascular endothelial cells (MVEC) and human embryonal fibroblasts (HEF) (**Chapter 2**). Membrane perturbation was detected in both types of endothelial cells with both virus strains but not in fibroblasts. Membrane effects were only found at 37°C and not at 4°C, which could mean that not only the binding to, but also the penetration of the virus into the endothelial cell is necessary to induce this effect. The virus strain adapted to endothelial cells, which yields a much higher infection level in HUVEC than AD169, failed to show a higher degree of membrane perturbation. This may indicate that the level of productive infection is not the determining factor for the induction of membrane perturbation. Fibroblasts do not play a central role in establishing the procoagulant / anticoagulant balance of the vascular wall as endothelial cells do and the differences in the CMV-induced procoagulant response between these two cell types might very well reflect this. It can be assumed that membrane perturbation facilitates the interaction of coagulation factors which makes the surface of the vessel more procoagulant, and may lead to a (pre) thrombotic state, one of the phenomena observed in arteriosclerosis.

It has previously been described that molecular changes that render endothelial cells more procoagulant in response to a HCMV infection include secretion of the antithrombotic factor VIII carrier protein, von Willebrand factor (Bruggeman et al., 1988) and tissue factor (Vercellotti et al., 1997) and rearrangement of the cell membrane phospholipids (Van Dam-Mieras et al., 1992). All of these responses could play a role in the development of vascular pathology associated with CMV disease. Furthermore, only a significant association between HCMV and atherosclerosis was found in individuals with high levels of lipoprotein(a) and

fibrinogen, indicating that HCMV infection alone may not be enough to cause atherosclerosis (Nieto et al., 1997). Therefore it is speculated that HCMV can enhance vascular disease as a co-factor, which is corroborated by our results.

Because there is a remarkable heterogeneity among endothelial cells, possibly due to the local conditions under which the endothelial cells differentiate (Garlanda and Dejana, 1997), it was investigated whether the endothelial cells from macro- and micro-vascular origin would respond differently to CMV infection. These experiments (**Chapter 2**) show that microvascular endothelium is more responsive to infection with HCMV than macrovascular endothelium with respect to membrane perturbation. This indicates that also in the *in vivo* situation responses to HCMV infection of endothelial cells depend on the location of these cells in the vascular system.

In the studies described in **Chapter 3** we investigated whether the CMV-induced procoagulant activity and/or membrane perturbation was caused by a direct effect of the virus or by an indirect effect. We demonstrated that the effects must be due to an indirect effect since the supernatant, containing no infective virus, could induce both effects. It seems that the infection of permissive cells such as fibroblasts by CMV induces the production and secretion of soluble factors which are responsible for the phenomena observed in HUVEC. It has already been shown by others that fibroblasts, infected with HCMV, release cytokines such as interleukin-8 (IL-8) and RANTES (Craig et al., 1997, Michelson et al., 1997). We therefore investigated if IL-1, tumour necrosis factor (TNF) and IL-8 could induce procoagulant activity and cause membrane perturbation in endothelial cells. We found that indeed all of these cytokines were able to evoke both responses. Therefore, we conclude that the virus probably induces procoagulant activity and causes membrane perturbation indirectly via the release of cytokines or other factors. Such an involvement of cytokines in virus induced vascular pathology is indeed what can be expected.

It has been reported, however, that purified HCMV virus *per se* can also evoke a procoagulant response. The purified HCMV constitutively displays phosphatidylserine (PS) and thereby constitutes a procoagulant surface on which coagulation factors interact to form a prothrombinase complex which generates thrombin (Prydzial and Wright, 1994). The HCMV envelope also contains tissue factor which contributes to the procoagulant activity (Sutherland et al., 1997). In this context we should of course realise that virus envelopes are complex structures in which the phospholipids are derived exclusively from the host cell, whereas the proteins are encoded by both the host and virus genome. A tissue factor homologue is not encoded by the HCMV genome and, therefore, must be acquired along with the procoagulant phospholipids from the host. As viral infection will result in cellular activation we can postulate that the HCMV surface will differ from that of quiescent

cells lining the vascular wall. The inner lining of the vascular wall normally is not in a procoagulant state, but such a state can be reached by activation. A control mechanism restricts the expression of procoagulant phospholipids to areas of vascular injury / activation (Bever et al., 1983). As HCMV is released from activated cells it can be assumed that the envelope of the virus reflects the surface of these activated cells, in their procoagulant state. It was found, however, that a preparation of dense bodies and non-infectious particles can also induce procoagulant activity. This means that a productive infection of the cells would not be necessary to induce procoagulant activity, since these particles are not capable of productively infecting cells. The question of course remains whether *in vivo* or *in vitro* the concentration of the viral particles reaches a level locally sufficient to induce a procoagulant response, without any other activation of the cells. *In vivo* will this probably not occur since such local virus concentrations will mainly occur intracellularly and are not found extracellularly. Our *in vitro* results show that no procoagulant activity can be detected when the penetration of the virus is inhibited by the incubation at 4°C during infection, which points to cellular activation in the establishment of the procoagulant response.

A procoagulant response induced by HCMV-infection can be seen as belonging to the defence strategy of the host against viral infection. Coagulation then could be perceived as supporting an inflammatory response.

Inflammatory responses, which are believed to play a crucial role in the onset of atherosclerotic vascular diseases (Ross, 1993), develop via the adhesion of leukocytes to the endothelium. Our group and others have found a release of IL-8, a potent neutrophil chemoattractant with monocyte chemotactic activity, in HCMV infected fibroblasts (Craig et al., 1997) In the study described in **Chapter 3** we describe the release of IL-8 by HCMV infected endothelial cells *in vitro*. The fact that HCMV induces an increase in the adherence of polymorphonuclear cells and monocytes (Span et al., 1991a, Span et al., 1991b) on endothelial cells, could be caused by the increase in expression of adhesion molecules like ICAM and ELAM and with the release of chemokines like IL-8 by infected endothelial cells.

HCMV infection of endothelial cells leads to quite distinctive effects, in spite of the fact that the productive infection level of HCMV is very low in these cells. The infection level of the HCMV laboratory strain AD169 in HUVEC is between 5 and 10%. In **Chapter 4**, we describe our study on the effect of activation of cellular signal transduction pathways on the productive HCMV infection of HUVEC. We found that the phorbol ester phorbol 12-myristate 13-acetate (PMA) increases this infection level, indicates that activated endothelial cells are more permissive for HCMV than quiescent cells. From our observation we conclude that, protein kinase C seems to

be a main factor involved in the PMA-stimulated increase in HCMV infection. Thus, from the results described in Chapters 3 and 4 it can be concluded that on the one hand CMV infection results in activation of endothelial cells, whereas on the other hand activation of endothelial cells can lead to an increase of HCMV infectivity.

During injury endothelial cells are activated by proinflammatory cytokines, like IL-1 and TNF and by reactive oxygen intermediates. The transcription factor NF- κ B plays an important role in the induction of genes during inflammatory responses. Activation of this transcription factor is triggered by a wide variety of proinflammatory peptides, cytokines, PMA as well as reactive oxygen intermediates like H₂O₂ (Munroe et al., 1995). Protein kinase C, an important factor in the PMA-stimulated increase in HCMV infection in endothelial cells, has been shown to activate NF- κ B (Ghosh and Baltimore, 1990), which itself stimulates transcription of HCMV (Löser et al., 1998, Prösch et al., 1995). Therefore, it can be hypothesised that the increase in HCMV infection level in endothelial cells after PMA stimulation is due to activation of NF- κ B, which enhances the transcription of HCMV genes. However, other mechanisms such as phosphorylation of receptors required for HCMV entry like the 92.5 kDa cell membrane receptor for HCMV glycoprotein gH, and the host cell annexin II, which can bind HCMV gB, may also be involved (Jost and Gerke, 1996, Keay and Baldwin, 1996, Pietropaolo and Compton, 1997).

Tissue injury causes cellular inflammatory responses during which T-cell, monocytes and neutrophils are recruited to the site of injury. The generation and release of reactive oxygen intermediates play a role in such processes. The generation and release of reactive oxygen intermediates may enhance HCMV infection in endothelial cells and smooth muscle cells via the activation of NF- κ B (Vossen et al., 1997, Scholz et al., 1996, Speir et al., 1996). The pro-inflammatory cytokine TNF is also able to activate NF- κ B. Thus, injury of endothelial cells may be capable of activating HCMV expression by the activation of NF- κ B.

HCMV infection, on the other hand, influences signal transduction pathways which have an effect on PKC activation (Nokta et al., 1987, Valyi-Nagy et al., 1988). Activation of PKC induces, among others, the expression of adhesion molecules on endothelial cells and is involved in the regulation of the endothelial permeability (Lane et al., 1989, Mason et al., 1997, Siflinger-Birnboim and Malik, 1996, Stasek and Garcia, 1992). This might lead to recruitment of inflammatory cells like monocytes and neutrophils to the site of infection followed by tissue injury.

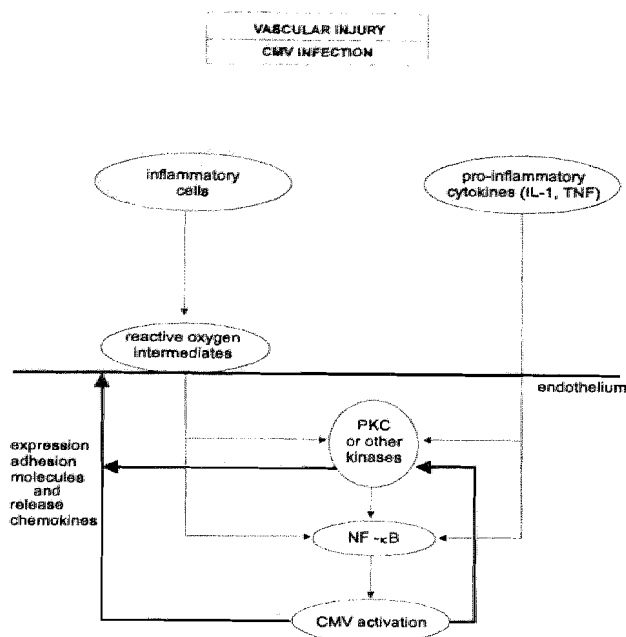


Fig. 1 Schematic representation of shared mechanisms activated by vascular injury and HCMV infection

Inflammatory mediators such as IL-1 and TNF also induce the expression of adhesion molecules, possibly through activation of signal transduction pathways such as the PKC pathway and by activating NF- κ B (Lane et al., 1990).

In conclusion, both vascular injury and HCMV infection activate the same defence and activation processes, namely the expression of adhesion molecules and the induction of procoagulant activity. Due to this phenomenon it is difficult to distinguish between association and causation as well as between the primary and secondary effect of this virus on vascular wall pathology. Furthermore, it should be kept in mind that the experiments were done *in vitro* and that the a straightforward extrapolation of results of *in vitro* experiments to the *in vivo* situation is never allowed.

Endothelial cells are infected only to a limited extent whereas fibroblasts are fully permissive. The mechanisms responsible for this difference have not been elucidated yet. In the last two Chapters of this thesis (**Chapters 5 and 6**) experiments on the differences between the early stages of HCMV infection in endothelial cells and fibroblasts are described. Endothelial cells are infected to only a limited extent whereas fibroblasts are fully permissive. We found that the expression of the IE antigen started later after infection in the low permissive

HUVEC than in the fully permissive HEF. This can be explained by the fact that transport of the virus to the nucleus, where viral transcription takes place, is slower in HUVEC than in HEF. In this respect the observation that viral DNA accumulates around the nucleus of HUVEC but not around those of HEF was most interesting. The entrance into the endothelial cells does not seem to be hampered, since viral DNA is detected in the cytoplasm of nearly all endothelial cells. These findings indicate that the virus cannot reach the nucleus of endothelial cells as efficiently as that of fibroblasts, which would lead to the low permissiveness of HUVEC for the HCMV strain AD169.

The actual mechanism of virus entry into the host cell could also contribute to the differences in virus infections of different cell types. It can be concluded from electronmicroscopic studies that HCMV enters both HUVEC and HEF but that the mechanism of entrance in HUVEC differs from that in HEF. The entry of HCMV in HUVEC appears to occur through endocytosis, while in HEF the virus enters the cell through fusion. The observation that, as a result of the entrance procedure, in HUVEC the virus is enclosed in vesicular structures while in HEF the virus occurs free in the cytoplasm, could implicate that the transport mechanism of the virus to the nucleus is different between the two cells types. The fact that cytochalasin B, an inhibitor of the F-actin polymerisation which is involved in cytoplasmatic transport processes, only influences the infectivity of HCMV in HUVEC and not in HEF could lend support to this hypothesis.

Another phenomenon contributing to the low permissiveness of HUVEC for the HCMV strain AD169 is degradation of the virus within the vesicular structures after viral entrance. Eight hours after infection the vesicular structures are degraded and no viral particles can be identified in the cytoplasm, which indicates that degradation of the virus in HUVEC may be a mechanism to protect the cell against HCMV infection. On the other hand, disassembly of the virus is necessary for the viral DNA transport into the nucleus and it is difficult to distinguish between viral degradation and the disassembly of the virus. This means that it will be necessary to investigate the enzymes involved in the degradation of the vesicle and the viral particles. The question that remains is why the viral entry is different in HUVEC and HEF.

Viral entry of the cell by endocytosis, the inefficient uptake of viral DNA by the nucleus as well as the presumed degradation of HCMV in HUVEC are interesting aspects of the interaction between virus and host. We can raise the question whether this sequence of events is orchestrated by the host cell or rather reflects a property of the virus. In this respect it would be interesting to repeat the experiments using an endothelial adapted HCMV virus strain in HUVEC, and to compare the

results obtained with that strain to our present results obtained with the AD169 strain.

Due to the crucial location of endothelial cells, it is attractive to assume that most of the cells protect themselves against productive CMV infection, and as a consequence the tissue lying behind this protective layer. However, even a low CMV infection grade of the endothelium can induce several responses like membrane perturbation, generation of procoagulant activity, release of cytokines and the expression of adhesion molecules, which implicates that CMV could play a role as a co-factor in various vascular diseases. The question whether CMV infection of endothelial cells and subsequent cellular activation is the primary cause of vasculopathy or if it is a co-factor in a multicausal process still remains.

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SAMENVATTING

SAMENVATTING

Humaan cytomegalovirus (HCMV) lijkt betrokken te zijn bij verschillende vasculaire aandoeningen, zoals transplantatie geassocieerde arteriosclerose, restenose en atherosclerose. Dit proefschrift beschrijft enkele mogelijke mechanismen waardoor de infectie van endotheelcellen door HCMV kan bijdragen aan deze vaatziekten.

In hoofdstuk 2 wordt de invloed van HCMV op endotheelcellen met betrekking tot membraanverstoringen en stolling, beide belangrijke mechanismen bij het ontstaan van vaat-aandoeningen, nader bestudeerd. Endotheelcellen, geïsoleerd uit de microcirculatie (MVEC) en macrocirculatie (HUVEC), vertonen een duidelijke dosisafhankelijke membraanverstoring kort na infectie met HCMV. De membraanverstoring wordt alleen gevonden als de endotheelcellen geïnfecteerd worden bij 37°C, wat een aanwijzing is dat binding van het virus aan de celmembranen alleen niet voldoende is om dit effect te veroorzaken. Het virus type, AD169 (een HCMV laboratorium stam welke gekweekt wordt op humane embryonale fibroblasten (HEF)) en VHL-E (een endotheel geïmpadateerde HCMV stam), waarmee het endotheel geïnfecteerd wordt is niet van invloed op de mate van membraanverstoring. Daarentegen is er wel een duidelijk verschil voor wat betreft de mate van infectie in endotheelcellen in vitro: AD169 geeft een lage infectiegraad in endotheelcellen, terwijl VHL-E een hoge infectiegraad geeft. Fibroblasten, die in vitro ook goed geïnfecteerd worden door HCMV vertonen geen significante membraanverstoring. Dit duidt erop dat de mate van infectie niet de bepalend factor is die de membraanverstoring veroorzaakt.

De vraag is of de membraanverstoring en stolling gevonden bij HCMV geïnfecteerde humane endotheel wordt door een indirect of een direct effect van het virus. Uit het feit dat het supernatant van het virus preparaat, waaruit het virus verwijderd is door middel van ultracentrifugatie, ook in staat is om stolling en membraanverstoringen te veroorzaken in tegenstelling tot het kweeksupernatant van ongeïnfecteerde fibroblasten, geeft aan dat mogelijk een indirect effect van het virus verantwoordelijk is voor deze fenomenen (Hoofdstuk 3). Het lijkt dus aannemelijk dat fibroblasten geïnfecteerd met HCMV naast het afgeven van virus ook substanties afgeven aan het kweeksupernatant, die verantwoordelijk kunnen zijn voor de geobserveerde effecten in endotheelcellen. Aangezien reeds bekend was dat onder andere cytokines door geïnfecteerde fibroblasten worden afgegeven, is de invloed van verschillende cytokines, zoals interleukine-1 (IL-1), IL-8 en tumor necrose factor (TNF), op de membraanverstoring en stolling onderzocht. Al deze cytokines, die ook tijdens een infectie in vivo geproduceerd worden, waren in staat om stolling en membraanverstoringen te induceren. Uit al deze gegevens kan geconcludeerd

worden dat de geïnduceerde stolling en membraanverstoringen indirect door het virus geïnduceerd worden, en dat de afgifte van cytokines of andere onbekende factoren na infectie van HCMV hiervoor verantwoordelijk kunnen zijn. Deze hypothese wordt versterkt doordat in Hoofdstuk 3 verder is aangetoond dat HCMV de afgifte van IL-8 kan induceren in HUVEC.

IL-8 is een chemoattractant voor polymorfonucleaire cellen en monocyten. Al eerder is aangetoond dat HCMV de expressie van adhesiemoleculen verhoogt en dat er na infectie een verhoogde hechting plaatsvindt van polymorfonucleaire cellen en monocyten aan endotheelcellen. De verhoging van de hoeveelheid hechtende cellen is waarschijnlijk het gevolg van de verhoging van de expressie van de adhesiemoleculen en de afgifte van chemokines zoals IL-8. Immunologische processen zoals ontstekingen worden onder andere in gang gezet door de hechting van leukocyten op het endotheel. Daarom kan gespeculeerd worden dat HCMV in staat is om immunologische processen te induceren die uiteindelijk kunnen leiden tot verschillende vasculaire aandoeningen.

Ondanks de duidelijke reactie van HUVEC op de infectie van de HCMV stam AD169 is de mate waarin deze cellen geïnfecteerd worden zeer laag: tussen de 5 en 10% van de endotheelcellen kunnen geïnfecteerd worden door HCMV. In Hoofdstuk 4 is beschreven dat de forbol ester forbol 12-myristate-13-acetaat (PMA) in staat is de mate van infectie in endotheelcellen significant te verhogen. Door gebruik te maken van diverse proteïne kinase activatoren en remmers is aangetoond dat de belangrijkste factor betrokken bij de door PMA verhoogde infectie proteïne kinase C (PKC) is. PKC is een enzym dat eiwitten fosforyleert en zo betrokken is bij vele cellulaire processen, zoals de activatie van de transcriptie factor NF- κ B en de expressie van adhesiemoleculen. In geactiveerde cellen is de PKC activiteit vaak verhoogd. Dit impliceert dat HCMV in staat is om geactiveerde endotheelcellen beter te infecteren dan cellen in rust en dat het virus gebruikt maakt van basale cellulaire processen.

Ondanks het feit dat PMA de infectie kan verhogen in endotheelcellen is het aantal geïnfecteerde cellen nog steeds een veel lager dan bij fibroblasten, waar bijna alle cellen geïnfecteerd worden. In de Hoofdstukken 5 en 6 van dit proefschrift is daarom het infectieproces van de HCMV stam AD169 in HUVEC bestudeerd en vergeleken met het infectieproces van deze HCMV stam in HEF. Hierbij zijn enkele duidelijke verschillen aangetroffen tussen het infectieproces in deze endotheelcellen en fibroblasten. **A** De expressie van de vroege (immediate early) en late (late) virale antigenen is vertraagd in endotheelcellen ten opzichte van fibroblasten. **B** Met behulp van in situ hybridisatie is een ophoping van viraal DNA geconstateerd rond de kernen van endotheelcellen 8 uur na infectie terwijl op dit zelfde tijdstip viraal

DNA in de kernen van de fibroblasten reeds aanwezig is. **C** Uit elektronenmicroscopische opnamen van HCMV geïnfecteerde endotheelcellen en fibroblasten bleek dat het mechanisme waarmee het virus de cel binnenkomt verschillend is in beide celtypen. Het virus komt via endocytosis endotheel cellen binnen terwijl het via fusie met de celmembraan fibroblasten binnen komt. Doordat het virus in endotheelcellen via endocytosis binnen komt bevindt het virus zich in deze cellen in vacuoles die afgebroken worden in het cytoplasma. Of het virus ook geheel afgebroken wordt, dus zowel op eiwit niveau als op DNA niveau, is nog niet duidelijk. Echter een normale virale structuur van het virus is 8 uur na infectie niet meer aantoonbaar met behulp van de elektronenmicroscopie. **D** Cytochalasin B, een remmer van de F-actine polymerisatie, remt alleen de infectie in endotheelcellen, wat aangeeft dat intacte actinefilamenten nodig zijn.

Deze verschillen tussen HUVEC en HEF geven aan dat de binnenkomst en het transport van AD 169 in HUVEC geheel anders is dan in HEF. De ophoping van het virale DNA en de afbraak van het virus in deze endotheelcellen zouden een verklaring kunnen zijn dat deze cellen slecht infecteerbaar zijn door AD169. Of deze verschillen veroorzaakt worden door verschillen tussen beide celtypen of een inherente eigenschap zijn van het virus is op dit moment nog niet duidelijk. Verwacht wordt dat experimenten met een endotheel-geadapteerde HCMV stam hierover meer duidelijkheid kunnen verschaffen.

Vanwege de strategische ligging van endotheelcellen, tussen het circulatie systeem en het omliggende weefsel, is het voor de hand liggend om aan te nemen dat endotheelcellen zich beschermen tegen de infectie met HCMV. Echter doordat de infectie van enkele endotheelcellen toch leidt tot duidelijk meetbare effecten zoals verhoogde stollingsactiviteit, verhoogde membraanverstoring, expressie van adhesiemoleculen en de afgifte van cytokines, kan verondersteld worden dat het cytomegalovirus een rol kan spelen bij verschillende vasculaire ziektes. Het is moeilijk om aan te tonen of cytomegalovirus infectie de oorzaak zijn van vasculaire pathologie of dat HCMV infectie van endotheelcellen eerder het gevolg is van immunologische processen die bij deze vasculaire afwijkingen een rol spelen. Verdere in vitro en in vivo studies zullen hierin meer duidelijkheid moeten verschaffen.